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13. ABSTRACT (Maximum 200 words) This project was aimed at learning more about the role of cysteine string proteins (csps) in the function of presynaptic calcium channels. An unexpected finding that emerged early in the course of this investigation is that csps are synaptic vesicle proteins. Our hypothesis is that csps mediate a novel interaction between synaptic vesicles, and presynaptic calcium channels that only permits these channels to open when there is a vesicle docked in the immediate vicinity of the channel. Studies we conducted using csp mutant Drosophila support this hypothesis. Thus, synaptic transmission is conditionally abolished in these mutants, but this presynaptic block of transmitter release can be overcome by reagents (like, calcium ionophores) that bypass the calcium channels and allow calcium ions to enter the nerve terminal and trigger secretion. We are now exploring the possibility that this modulatory action of csps is mediated via G-proteins (since we have obtained no evidence of a direct interaction between csps and presynaptic calcium channels), and thereby expect to obtain a better understanding of this interaction at a molecular level.			
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PRESYNAPTIC CALCIUM CHANNELS: cDNA CLONING

FINAL REPORT

dates covered: 2/15/93-2/14/97

by: Cameron B. Gundersen, Ph.D.

1 May 1997

U.S. ARMY RESEARCH OFFICE

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FINAL REPORT

DAAH04-93-G-0051

A) STATEMENT OF THE PROBLEM STUDIED:

The original goal of this proposal was to clone cDNAs encoding subunits of presynaptic calcium (Ca) channels. These subunits were then to be expressed in frog oocytes as a means of learning more about the properties of these channels. In particular, we were interested in the functional role of a novel, putative Ca channel subunit that we had identified in the electric fish, *Torpedo* (Gundersen & Umbach, 1992) and named CCCS1 (for candidate Ca channel subunit 1). This protein has been subsequently re-named *Torpedo* cysteine string protein (csp), because of its high sequence similarity to a protein in *Drosophila* that had been shown to reside almost exclusively at synapses (Zinsmaier, et al., 1990). The thrust of our planned investigations changed early in the award period due to two developments. First, several other groups in rapid succession reported the cloning of cDNAs encoding various subunits of different sub-types of Ca channels, including subunits of Ca channels that were likely to reside at nerve endings. This provided us with some of the requisite tools to conduct the planned physiological experiments, without first having to clone the necessary cDNAs. Second, we made the unexpected observation that csps are synaptic vesicle proteins (Mastrogriacomo, et al., 1994). This result eliminated the possibility that csps were novel subunits of presynaptic Ca channels, and instead suggested that these proteins might mediate a novel regulatory interaction between synaptic vesicles and presynaptic Ca channels. It has been a major goal of our efforts to test the hypothesis that csps participate in a regulatory link between synaptic vesicles and presynaptic Ca channels. The following exposition focuses on details of our efforts to understand this process both at a physiological level, as well as at a biochemical level. In addition, I begin by recapitulating the largely negative results of experiments we have conducted to try to clarify csp function by co-expressing it in oocytes along with Ca channel cDNAs.

B) SUMMARY OF THE MOST IMPORTANT RESULTS

1) Co-expression Experiments using *Xenopus* Oocytes

The initial hypothesis that we tested was the possibility that co-expression of csp cRNA along with cRNA encoding known Ca channel subunits (in particular, we focused on subunits encoding either N-type or P-type Ca channels, due to the data implicating these channel types in presynaptic function at selected synapses) would modify the properties of the expressed channels. Because our initial experiments showed that csp antisense cRNA suppressed the expression of N-type Ca channels (Gundersen & Umbach, 1992), we inferred that co-expression of csps might lead to more-robust expression of Ca channels in the oocytes. However, none of our experiments showed any direct effect (on channel properties, like peak current, steady-state inactivation or voltage dependence of gating) of co-expressing csp cRNA in oocytes along with Ca channel cDNAs. Similarly, we supplied csp clones to a couple of other groups for similar investigations, and no positive results were obtained. From these experiments, it appears that if there is a csp-Ca channel interaction, it either requires one or

more additional proteins, or it is not faithfully assembled in oocytes. As I have pointed out in some of the progress reports, there is reason to believe that the csp-Ca channel interaction may involve other proteins. Thus, we are using biochemical approaches in an effort systematically to identify csp-interacting proteins that are candidates to mediate this interaction. Any candidate proteins identified biochemically (see below) will then be tested for activity by co-expression in oocytes.

2) Csp Function Revealed by Studying *Drosophila csp* mutants

With the original indication that csps play some role in modulating the function of N-type Ca channels expressed in frog oocytes (Gundersen & Umbach, 1992), we predicted that if it were possible to obtain *csp* mutant alleles of *Drosophila* (that were not early embryonic lethals), these mutants should show alterations in transmitter release that should reflect altered Ca channel function at the nerve terminal. A consortium of investigators, that included the original discoverer of *Drosophila* csps, was successful in obtaining *csp* mutant alleles (see, Zinsmaier, et al. 1994). These mutants displayed several interesting traits. First, there was a high degree of embryonic lethality, but in all mutant strains that were isolated, there were some survivors (Zinsmaier, et al., 1994). Among those mutant embryos that hatched, it was found that larval forms, as well as adult flies, were temperature-sensitive paralytics. Thus, with as little as an eight degree step above room temperature, *csp* mutant organisms stopped moving. With prolonged temperature challenge (>1h), these organisms died. However, regardless of the ambient temperature, *csp* mutants died prematurely (survival time is less than 6 days for adult *csp* mutants, compared to 40-50 days for wild-type organisms). These observations (all reported in Zinsmaier, et al. 1994), led us to explore the cellular basis of the temperature-dependent paralysis of these mutants.

Drosophila larvae are well suited for studies of neuromuscular transmission, so we examined the efficacy of synaptic transmission in *csp* mutants at both permissive temperature (22° C), as well as at non-permissive (or restrictive) temperature (30°C). The results showed convincingly that these mutants suffered a failure of evoked transmitter release at non-permissive temperature (Umbach, et al., 1994). Because spontaneous transmitter release (that produces miniature excitatory junctional potentials, or mejps) was still intact in the *csp* mutants, we inferred that the terminal steps of the exocytotic event were not abolished in *csp* mutants (Umbach, et al., 1994). Instead, there was a complete failure of nerve impulses to drive the normal secretion of transmitter in these mutants. While these results are compatible with the idea that Ca channels may not open normally (indeed, they may not open at all!) in these mutants at restrictive temperature, they do not exclude other explanations for these results (though, I should mention that we did exclude trivial explanations for this transmission failure; thus, there was no change in post-synaptic sensitivity to glutamate, and we also showed that nerve impulses still propagated into the terminals of these mutants). The foregoing investigations were a prelude to some pharmacologically oriented studies that were aimed at clarifying the locus of the lesion in synaptic transmission in *csp* mutants.

To summarize these pharmacological results, our data point strongly to the conclusion that presynaptic Ca channels fail to open properly in response to nerve terminal depolarization in *csp* mutants at restrictive temperatures (Umbach & Gundersen, 1997). This conclusion derives from the fact that both the spider venom toxin, alpha-latrotoxin, and tha Ca-ionophore,

ionomycin, bypass the failure of secretion in *csp* mutants at elevated temperatures. However, raising the concentration of either Ca ions or K ions (which normally depolarizes nerve terminals and promotes a high frequency discharge of mejsps), does not overcome the secretory failure in these mutants. Similarly, the drug, 4-aminopyridine, (which usually chemically potentiates quantal transmitter release by prolonging the depolarization of the nerve terminal, thereby facilitating Ca ion entry via open Ca channels) does not circumvent the transmission failure in *csp* mutants. These latter three strategies (increased bath Ca or K, or the addition of 4-aminopyridine) will only potentiate transmitter release if Ca channels are capable of opening and conducting Ca ions in the first place. The failure of all three strategies to promote transmitter release, suggests that the Ca channels are not opening in the first place. The ability of Ca ionophore to bypass these presumably defective channels and deliver Ca ions to trigger transmitter secretion supports this idea. Thus, we conclude that under normal circumstances there is some type of regulatory coupling between presynaptic Ca channels and the vesicle(s) that dock in their vicinity. Csps presumably are important for this interaction to the extent that in the *csp* mutant *Drosophila*, this interaction breaks down at elevated temperature, leaving synaptic transmission (and, the flies) paralyzed. We are currently developing optical imaging methodology to monitor cytosolic ionized calcium in these insect nerve endings as a means of assessing more directly the apparent failure of Ca channels to open in *csp* mutants.

3) Biochemical Investigations of Csp-Ca Channel Interactions

We began these efforts by looking for evidence of proteins that co-immunoprecipitate with csps. Unfortunately, all of our anti-csp antibody preparations have proven to be relatively inefficient at immunoprecipitating csps. Nevertheless, we have tested whether these antibodies co-immunoprecipitate Ca channels, by first labeling these channels with ligands, like omega-conotoxin. Regardless of our efforts in this context, we have found no evidence for a direct interaction of csps and these channel proteins. However, we did obtain evidence that csps co-immunoprecipitated with Hsp70 immunoreactive protein. This result was un-surprising, given that csps have a J-domain, which in other proteins binds Hsp70. However, we do not know whether this Hsp70 protein is important for csp's presumed interaction with presynaptic Ca channels. This is still an issue that we are studying.

Recently, there has been another intriguing development that we are pursuing in the context of csp-Ca channel interactions, and this was the recent report of another protein, GAIP, that has a cysteine string (deVries, et al., 1996). Interestingly, GAIP is a member of a family of proteins (the RGS proteins) that regulates the GTPase activity of heterotrimeric G-protein alpha subunits. One of the defining features of the consensus sequence of RGS proteins is a highly conserved pair of amino acid residues (EN), and many RGS family members have an EEN residue triplet. Interestingly, csps (from insects to man) have an EEN triplet between their cysteine string and the J-domain. Thus, we are exploring the possibility that csps constitute a novel class of RGS-like proteins. We are currently setting up to test whether csps bind to G-protein alpha subunits (like, RGS proteins), and if they do, we will examine whether this binding alters either the rate of GTP hydrolysis, or GDP exchange. These results may provide us with useful insights into the mechanism by which csps modulate Ca channels.

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Zinsmaier, K.E., Eberle, K.K., Buchner, E., Walter, N. & Benzer, S. (1994) Science 263, 976-979.

C) LIST OF ALL PUBLICATIONS RESULTING FROM THIS GRANT

1) Mastrogiacomo, A., Evans, C. & Gundersen, C.B. (1994) Antipeptide antibodies against a Torpedo cysteine string protein. J. Neurochem. 62, 873-880

2) Swanson, G.T., Umbach, J.A. & Gundersen, C.B. (1994) Glia of the cholinergic electromotor nucleus of Torpedo are the source of the cDNA encoding a GAT-1-like GABA transporter. J. Neurochem. 63, 1-12.

3) Umbach, J.A. & Gundersen, C.B. (1997) Evidence that cysteine string proteins regulate an early step in the calcium-dependent secretion of neurotransmitter at *Drosophila* neuromuscular junctions. J. Neuroscience (to be submitted).

D) PARTICIPATING SCIENTIFIC PERSONNEL

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